Response to the Final Office Action:

A. Status of the Claims

Claims 1-19 and 29-38 were pending upon the issuance of the Final Office Action dated August 11, 2004. Claims 1, 2, 8, 9, 13, 15-19, 30, 32, 36, and 37 have been amended, claims 39-40 have been added, and claims 31 and 35 have been canceled. Support for the amendments and the new claims can be found throughout the specification and claims as originally filed. Claims 1-19, 29-30, 32-34, and 36-40 are therefore currently pending.

B. The Enablement Rejection Is Overcome

Claims 1-19 and 29-38 are rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. In summary, the Action contends that the specification does not enable the full scope of the subject matter of claims 1-19 and 29-38. From this, the Action states that undue experimentation would be required.

Applicants disagree with this rejection, claims 1-19 and 29-38 are enabled, and these claims satisfy all of the requirements of 35 U.S.C. § 112, first paragraph.

In an effort to obtain claims encompassing commercially relevant subject matter at this time, however, Applicants note that current independent claims 1 and 15 have been amended at the suggestion of the Action. The Action indicated that these revisions would overcome the present enablement rejection. *See* the Action, Page 10. The present enablement rejection is therefore rendered moot. Applicants reserve the right to pursue the subject matter of the originally filed claims in a future continuing application.

Applicants request that the present enablement rejection be withdrawn.

C. The Indefiniteness Rejection Is Overcome

The Action rejects claims 1-19 and 29-38 under 35 U.S.C. § 112, second paragraph, as being indefinite. Specifically, the Action contends that the term "the lymph node" in claims 1 and 15 does not contain a proper antecedent basis. Applicants note that claims 1 and 15 have been amended to correct any antecedent basis issues. This rejection is therefore rendered moot and should be withdrawn.

D. The Obviousness Rejection Is Overcome

1. Summary of the Rejection

The Action rejects claims 1-14, 29, and 31-34 under 35 U.S.C. § 103(a) as being unpatentable over the teachings of Allen *et al.* in view of either Griffiths or U.S. Patent No. 5,420,105 to Gustavson *et al.* and in further view of Oussoren (1997). The Action contends that Allen *et al.* discloses a method of delivering a first composition comprising a ligand conjugated to a tumor specific antibody and delivering a second composition comprising an anti-ligand/therapeutic agent/diagnostic agent containing liposomal carriers. The Action, page 14. The Action admits, however, that Allen *et al.* fails to teach that:

> … the liposomal particle can also be used to enhance the delivery of the first composition comprising a tumor specific antibody conjugated to a ligand such as biotin to one or more targeted lymph nodes and that an anti-ligand/therapeutic compound/liposomal complex is administered separately, whereby the biotin binds to avidin prior to their entry into the one or more targeted lymph nodes… .

The Action, page 15. It is also conceded by the Action that Allen *et al.* fails to disclose "that a dye can be used to enhance the visualization of the delivered agents in a treated subject." *Id.*

To supplement the deficient teachings of this reference, the Action cites to Gustavson *et al.* and Griffiths and contends that these secondary references disclose the use of a colloidal based system is effective to increase the targeting of both a ligand conjugate or a subsequent anti-ligand conjugate at an intended target site such as a tumor site. The Action cites to

Oussoren (1997) and contends that it discloses that colloidal particles up to about .4 um in diameter are transported from an injection site into the lymphatic capillaries and localized in regional lymph nodes. From this, the Action concludes that it would have been obvious for one of ordinary skill in the art to employ a colloidal based delivery carrier to enhance the delivery and binding of ligand/anti-ligand at a target tumor site to which the first delivered ligand is bound in the bloodstream. *Id.* at page 15-16.

Applicants traverse the obviousness rejection. Claims 1-14, 29, and 31-34 are not rendered obvious over the cited references.

2. The Standard for Establishing a *Prima Facie* Case of Obviousness

It is well settled that "[t]he examiner bears the initial burden of factually supporting any *prima facie* case of obviousness. If the examiner does not produce a *prima facie* case, the applicant is under no obligation to submit evidence of non-obviousness." *Manual of Patent Examining Procedure* (MPEP) § 2142 (8th Ed. Rev. 1, 2003).

To establish a *prima facie* case of obviousness, the Examiner must show: (1) some suggestion or motivation, either in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to modify the reference or to combine reference teachings; (2) a reasonable expectation of success; and (3) the prior art reference teaches or suggests all of the claim limitations. MPEP § 2142; *see also In re Vaeck*, 947 F.2d 488. If any one of the three elements is missing, a *prima facie* case of obviousness cannot be established.

3. Summary of the Presently Rejected Claims

Applicants presently claim "[a] method for delivery and retention of an active agent in one or more targeted lymph nodes, comprising: a) injecting into a mammal a first composition comprising a ligand conjugated to a colloidal particle comprising a diameter of less than 500 nm;

and b) injecting into said mammal a second composition comprising an anti-ligand, wherein said anti-ligand binds to said ligand, wherein the active agent is bound to or encapsulated in either said colloidal particle or said anti-ligand, and wherein the anti-ligand aggregates with the colloid-ligand complex at, or just prior to reaching, the one or more targeted lymph nodes. Claim 1.

4. The Cited References Fail to Teach or Suggest Every Element of the Presently Claimed Invention

A necessary element in establishing a *prima facie* case of obviousness requires a showing by the Action that every element of Applicants' claimed invention is disclosed by the cited references. This has not been done.

i. The cited references fail to teach the claimed element of "wherein the anti-ligand aggregates with the colloid-ligand complex at, or just prior to reaching, the one or more targeted lymph nodes."

Neither the primary reference (Allen *et al.*), the secondary references (Griffiths and Gustavson *et al.*), or the tertiary reference (Oussoren (1997)) teach "wherein the anti-ligand aggregates with the colloid-ligand complex **at, or just prior** to reaching, the one or more targeted lymph nodes." Claim 1 (emphasis added).

Allen *et al.*, for example, appears to be directed towards methods of delivering an anti-tumor compound to a targeted tumor. *See* Allen *et al.*, the Title and Abstract. The method appears to include, first, an injection of an antibody modified by a ligand molecule to a subject. *Id.* at col. 12, lines 17-26. This "permits selective localization of the antibody to a target site." *Id.* Subsequently, "liposomes containing a liposome-entrapped compound, and a surface-bound anti-ligand molecule, such as avidin, are administered parenterally." *Id.* The Allen *et al.* reference, therefore, appears to teach that the aggregation of the modified antibody and the liposome occur **after** the binding to or entry into the targeted site.

The present claims, by stark contrast, are directed towards embodiments of the invention "wherein the anti-ligand aggregates with the colloid-ligand complex **at, or just prior** to reaching, the one or more targeted lymph nodes." The aggregation, therefore, takes place "at, or just prior" to reaching the lymph node. If anything, the Allen *et al.* reference teaches away from Applicants' claimed invention—it teaches the aggregation of two compounds **after** binding to or entry into the targeted site. *See Tec Air, Inc. v. Denso Mfg. Michigan, Inc.*, 192 F.3d 1353, 1360 (Fed. Cir. 1999) (noting that if "a person of ordinary skill in the art, upon reading the reference, would be ... led in a direction divergent from the path that was taken by applicant" the reference is said to "teach away."). A reference that "'teaches away' from the claimed invention is a significant factor to be considered in determining obviousness." MPEP § 2146.

The teachings of the secondary and tertiary references also appear to be similarly deficient. Griffiths, for example, is directed towards "methods of detecting and/or treating lesions in a patient." Griffiths, Abstract. The method appears to include the parenteral injection of three separate compositions—a targeting composition that **first** binds to a target lesion, a clearing composition that binds to the targeting composition, and a detection or therapeutic composition that binds to the clearing composition. *Id.* Similar to Allen *et al.*, the aggregation in Griffiths appears to be **after** binding to or entry into the targeted site.

The Gustavson *et al.* reference also appears to teach aggregation after binding to or entry into the targeted site. For example, Gustavson states:

> When one member of a ligand-anti-ligand pair (e.g., an anti-ligand) is localized to a target site via a targeting moiety such as a monoclonal antibody or the like, the binding pair member (anti-ligand) serves to target a subsequently administered complementary binding pair member (ligand)-active agent conjugate to target sites characterized by previously localized targeting moiety-binding pair member (anti-ligand). Such pretargeting methods useful in both diagnostic and therapeutic applications are also discussed.

Gustavson, col. 3, lines 5-15. In other embodiments, Gustavson *et al.* describes an already conjugated "targeting protein/polymeric carrier/drug conjugates... ." *Id.* at col. 2, lines 57-63. There does not appear to be any teaching in Gustavson *et al.* of aggregation "at or just prior to at, or just prior to reaching, the one or more targeted lymph nodes."

As for the tertiary reference, Oussoren (1997), it appears to concern lymphatic uptake of liposomes after subcutaneous injection. *See* Oussoren (1997), Abstract. The data presented in Oussoren (1997) does not appear to be related to the liposomal compositions aggregating with a second composition—much-less such aggregation occurring "at or just prior to reaching" a targeted site. Additionally, the data in Oussoren (1997) appears to indicate minimal liposomal retention in the lymph node. This is confirmed by the same collaborators in Figures 2(b) of *Oussoren and Storm, Advanced Drug Delivery Reviews*: 50;143-156 (2001) ("Oussoren (2001)), a copy of which is attached as Appendix A for the convenience of the Examiner. By contrast, Applicants' specification provides surprising and unexpected data that shows at least an 11-14 fold increase in liposomal retention in the lymph nodes. *See, e.g.*, the Specification at Tables 1-3 on pages 39-42, respectively. A detailed discussion of this data is presented in sections D5 and D6, below.

A commonality of all of the cited references is that that they appear to teach aggregation of two or more compounds **after** binding to or entry into the targeted site. There does not appear to be any teaching or suggestion in any of these references of Applicants' claimed element of "wherein the anti-ligand aggregates with the colloid-ligand complex **at, or just prior** to reaching, the one or more targeted lymph nodes." The cited references, therefore, fail to teach or suggest every element of Applicants' claimed invention—a necessary requirement to maintain

the present obviousness rejection. The present obviousness rejection should therefore be withdrawn.

Further, it appears that the Action contends that the limitation "wherein the anti-ligand aggregates with the colloid-ligand complex at, or just prior to reaching, the one or more targeted lymph nodes" is a functional limitation. This is improper for at least two reasons. First, the present claims are method claims—claims that describes methods of doing or achieving something. The claims are not composition claims. Second, even if the limitation is functional, the use of functional limitations is a well-accepted practice under U.S. patent law. *See* MPEP § 2173.05(g) ("There is nothing inherently wrong with defining some part of an invention in functional terms. Functional language does not, in and of itself, render a claim improper") (emphasis added) (citing *In re Swinehart*, 439 F.2d 210 (CCPA 1971)). The MPEP, in fact, states that "[a] functional limitation must be evaluated and considered, just like any other limitation of the claim, for what it fairly conveys to a person of ordinary skill in the art in the context in which it is used." *Id.*

ii. All of the cited references fail to teach or suggest "[a] method for delivery and retention of an active agent in one or more targeted lymph nodes."

None of the cited references appear to teach or suggest "[a] method for delivery and retention of an active agent in one or more targeted lymph nodes." For example, Allen *et al.* appears to be concerned with the treatment of solid-tumors. *See* Allen *et al.*, the Title and Abstract. Allen *et al.* does not appear to even mention lymph nodes. As for Griffiths, it appears to be directed towards the detection and therapy of lesions with biotin/avidin polymer conjugates. Griffiths, Title and Abstract. Gustavson *et al.* appears to disclose polymeric carriers for non-covalent drug conjugation. Gustavson *et al.*, Abstract. Oussoren appears to concern lymphatic uptake of liposomes after subcutaneous injection. *See* Oussoren, Abstract.

All of the cited references, therefore, fail to teach or suggest "[a] method for delivery and retention of an active agent in one or more targeted lymph nodes." The present obviousness rejection should therefore be withdrawn.

5. There Is No Motivation to Combine or Modify the Cited References

A second element necessary to establish a *prima facie* case of obviousness requires a showing by the Action of a motivation to combine or modify the teachings of the primary reference (Allen *et al.*) with those of the secondary references (Griffiths and Gustavson *et al.*) and the tertiary reference (Oussoren (1997)). This has not been done.

The examiner, for example, has cited to the Oussoren (1997) reference for the proposition that:

> ...one would have reasonably expected that the liposomal composition of Allen is localized and retained at any tumor site in a leukemia patient such as those residing in lymph nodes because of the evidences and teachings provided by Oussoren, which clearly teaches that colloidal particles up to about .4 um in diameter are transported from an injection site into the lymphatic capillaries and localized in regional lymph nodes (Figure 3).

The Action, page 17. This proposition is incorrect; a person of ordinary skill in the art would not have expected that the liposomal composition of Allen would have been retained in the lymph nodes based on the data that is presented in Figure 3 of Oussoren (1997). The data in Oussoren (1997), in fact, appears to indicate that the opposite is true—that there is minimal, if any, liposomal retention in the lymph nodes. This is shown in at least two instances: (1) the method of how the data is presented in Oussoren (1997) is misleading; and (2) the data that is presented by the same collaborators in Figures 2(b) of *Oussoren and Storm, Advanced Drug Delivery Reviews*: 50;143-156 (2001) ("Oussoren (2001)") shows that no more than 1.0-1.5% of the injected dose is retained in the lymph nodes.

The data that is presented in Figure 3 of Oussoren (1997) is presented in % of injected dose/gram in the regional lymph nodes; it is **not** presented in % of injected dose that is actually retained in the lymph nodes. The % of injected dose/gram in the regional lymph nodes is calculated by dividing the radioactive counts per minute taken up by lymph node by the gram weight of the lymph node. The obtained number is then divided by the total radioactive counts per minute actually injected in the animal and multiplied by 100. As can be seen, the calculation is dependent on the weight of the lymph node. Stated another, way, if the lymph node is small, it will appear that more liposomes are retained in the lymph node. By contrast, if the lymph node is large, it will appear that less liposomes are retained in the lymph node.

Therefore, the data that is presented in Figure 3 of Oussoren (1997) shows, for example, approximately 125% of injected dose/gram was recovered from the regional lymph nodes of a liposome particle having a mean size of 0.07μm. This data corresponds to approximately 1%-1.5% of the injected dose being retained and recovered in the lymph nodes. This is confirmed by the same collaborators in Figure 2(b) Oussoren (2001).

By contrast to both Oussoren references, however, Applicants' specification presents surprising and unexpected results that shows about an 11-14 fold increase in liposomal retention in the lymph nodes when a second anti-ligand composition is also injected. *See* Applicants' specification, Tables 1-3 on pages 39-42, respectively. This surprising and unexpected data is strong evidence of non-obviousness. *See, e.g., In re Pravin*, 54 F.3d 746, 750 (Fed. Cir. 1995) ("One way for a patent applicant to rebut a prima facie case of obviousness is to make a showing of 'unexpected results,' i.e., to show that the claimed invention exhibits some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or

unexpected."). This is especially true where the cited reference (Oussoren (1997)) teaches minimal liposomal retention in the lymph nodes.

Applicants also note that because all of the references appear to be directed towards the aggregation of two or more compounds **after** binding to or entry into the targeted site, the modification of these references to include Applicants' claimed element of "wherein the anti-ligand aggregates with the colloid-ligand complex **at, or just prior** to reaching, the one or more targeted lymph nodes" would render the cited references unsatisfactory for their intended purpose. *See* MPEP § 2143.01 (noting that a proposed modification cannot render the prior art unsatisfactory for its intended purpose or change the principle of operation of a reference).

Because of the apparent failure of the cited references to disclose any suggestion or motivation to combine or modify their teachings, a necessary element in establishing a *prima facie* case of obviousness has not been established. For at least this reason, the present obviousness rejection cannot be maintained.

6. There Is No Reasonable Expectation of Success that the Combination of the Cited References Would Work

An additional and independent element necessary to establish a *prima facie* case of obviousness requires a showing of a reasonable expectation of success that combining the teachings of the references would work. This also has not been done by the Action.

As noted directly above, the data that is presented in Oussoren (1997) and Oussoren (2001) confirms that there is minimal liposomal retention in the lymph nodes. Based on at least this data, a person of ordinary skill in the art would not have a reasonable expectation of success of Applicants' claimed invention. If anything, the data that is presented in the Oussoren references actually teaches away from Applicants' claimed invention; a person of ordinary skill reviewing the data in the Oussoren references would be discouraged from using liposomes to

delivery a target agent to the lymph nodes because of the apparent minimal liposomal retention in the lymph nodes. *See* Oussoren (1997), Figure 3, and Oussoren (2001), Figure 2b; s*ee also In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994) ("[a] reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference ... ."). The fact that the Oussoren references teach away from Applicants' claimed invention is strong evidence of non-obviousness. *See* MPEP § 2145 ("A prior art reference that 'teaches away' from the claimed invention is a significant factor to be considered in determining obviousness ... .").

Because all of the necessary elements required to establish a *prima facie* case of obviousness have not been established by the Action, the present obviousness rejection cannot be maintained. The obviousness rejection for claims 1-14, 29, and 31-34 should be withdrawn.

E. Comments Regarding the Previously Issued Species Election Requirement

The Examiner issued a Species Election Requirement in this case on October 6, 2003. Applicants believe that the present generic claims are allowable. Upon the allowance of a generic claim, Applicants are entitled to consideration of claims to additional species which are written in dependent form or otherwise include all the limitations of an allowed generic claim. *See* 37 C.F.R. § 1.141(a).

F. Conclusion

Applicants believe that the present document is a full and complete response to the Final Office Action dated August 11, 2004.

1. Introduction

The lymphatic systems comprises a network of lymphatic vessels and lymph nodes throughout the body. The lining of lymphatic vessels consists of very thin endothelium and intercellular junctions which allow absorption of interstitial fluid containing macromolecules (proteins) and particulate cellular matter [1]. Unfortunately, this route of entry to the lymphatics is also utilized by tumor cells and pathogens, such as bacteria and viruses. For example, many human cancers spread via and form secondary tumors in the lymphatic system [2]. The presence and replication of the human immunodeficiency virus in macrophages of lymphoid tissue at all stages of the acquired immunodeficiency syndrome (AIDS), suggest that lymph nodes play an important role in the propagation of HIV infection [3,4]. For treatment of diseases with lymphatic involvement it is desirable to develop approaches to deliver diagnostic, therapeutic and immunomodulatory agents to lymph nodes [5–8].

The function of the lymphatic system in the clearance of excess fluid and particulates from the interstitial tissue has generated interest in the use of colloidal systems for the targeting of agents to regional lymph nodes after local parenteral administration such as subcutaneous (s.c.), intramuscular (i.m) and intraperitoneal (i.p.) injection. The s.c. route of administration has been most extensively investigated for the lymphatic targeting of liposomes. Among the colloidal systems proposed for lymphatic targeting, liposomes have attracted considerable attention. The first observation of targeting of liposomes to the lymphatics was made when an i.v. injection of liposomes was accidentally administered interstitially [9]. Since then a limited number of reports have appeared in the 1980s on the lymphatic disposition of liposomes after local parenteral administration [10–13]. These studies mainly focused on the fate of the drug rather than on the fate of the liposomal carrier itself. More recently, experimental attention was given to the fate of the liposomal carrier itself.

The present paper intends to review the recent literature that appeared on the lymphatic absorption and lymph node uptake of liposomes after s.c. injection. Factors influencing lymphatic disposition after s.c. injection, such as injection site, liposomal size, lipid composition and lipid dose are addressed. The mechanism of localization of liposomes in regional lymph nodes is discussed as well. Additionally, attention is paid to the diagnostic and therapeutic potential of s.c. injected liposomes for diseases with lymphatic involvement.

2. Factors influencing lymphatic absorption and lymph node uptake

Subcutaneous administered liposomes do not have direct access to the bloodstream. Instead, they are taken up by lymphatic capillaries draining the injection site or remain at the site of injection (Fig. 1). Generally, lymphatic absorption occurs over the first 12 h after injection. After this initial period the absorption process is completed (Fig. 2A). Once the liposomes have traversed the interstitium and entered the lymphatic capillaries, they pass through the lymphatic system where they can be captured in regional lymph nodes. About 1–2% of the injected dose is taken up by regional lymph nodes. The time-frame over which lymph node uptake occurs is in line with the observed time frame of lymphatic absorption, i.e. the initial 12 h after injection (Fig. 2B). When expressed as percentage of injected dose per gram tissue, lymph node uptake of small neutral liposomes in regional lymph nodes is substantially higher (30–40-fold) than uptake in spleen and liver, the natural target organs for circulating liposomes (Fig. 3) [14]. Liposomes that pass through the lymphatics without being captured in lymph nodes reach the general circulation where they behave as if administered by the intravenous (i.v.) route. Lipo-

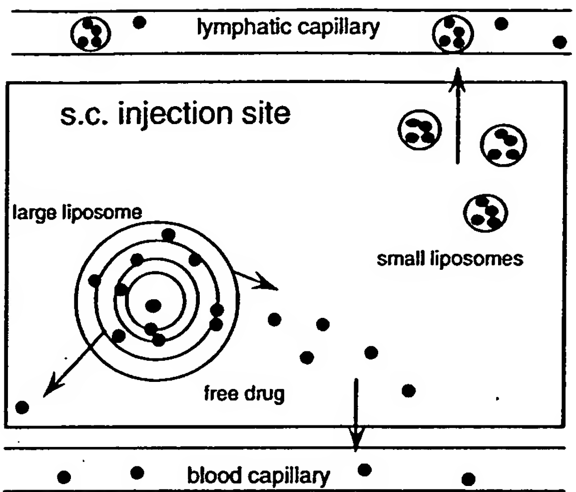

Fig. 1. Schematic representation of lymphatic absorption of s.c. injected liposomes. After s.c. injection liposomes do not have direct access to the bloodstream as the permeability of blood capillaries is restricted to water and small molecules. Instead, liposomes can be absorbed by lymphatic capillaries draining the s.c. injection site. Lymphatic absorption depends strongly on liposome size. Small liposomes (roughly <0.1 μm) can enter the lymphatic capillaries and accumulate in regional lymph nodes. Larger liposomes that remain at the injection site will slowly destabilize and degrade in time, concurrently losing their drug content. After release, free drug enters the blood circulation. Small molecular weight drugs (<16 kDa) will enter the blood compartment by passing through the pores in the blood capillary walls, whereas larger molecules are mainly transported by the lymphatics.

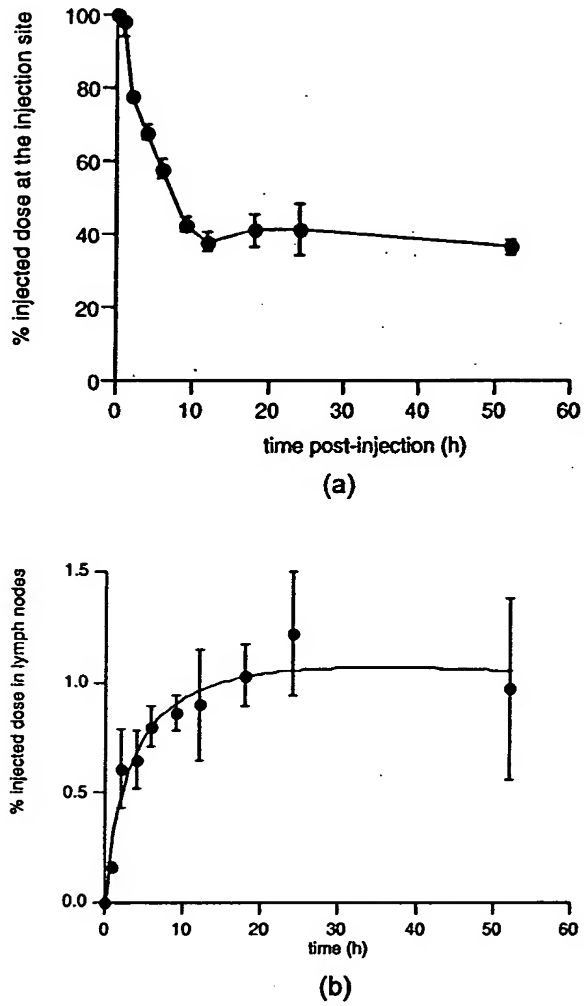

Fig. 2. Lymphatic absorption and lymph node uptake of a single dose of s.c. administered liposomes (EPC:EPG:Chol, 10:1:4 molar ratio, mean diameter 0.07 μm, 2.5 μmol lipid). A single dose of liposomes was injected s.c. into the dorsal side of the foot of rats. Recovery of liposomal label was determined at several time-points post-injection. (A) Percentage of injected dose recovered from the s.c. injection site. (B) Percentage of injected dose recovered from regional lymph nodes. Values represent the mean percentage±S.D. of four animals.

somes that remain at the injection site will slowly destabilize and degrade in time, concurrently losing their drug content. The actual drug concentration built up in the regional lymph nodes depends mainly on the degree of absorption of liposomes from the s.c. injection site and lymph node uptake. In this section the current insight into the factors influencing lymphatic absorption and lymph node uptake of s.c. injected liposomes are presented.

2.1. Influence of liposome size

Liposome size is the most important factor that determines the extent of liposome absorption of from the injection site after s.c. injection [15–17]. For small (<0.1 μm), neutral liposomes, the degree of lymphatic absorption can reach levels up to 70% of the injected dose (Fig. 4A) [14]. This size-dependency of absorption is likely to be related to the process of particle transport through the interstitium. The structural organization of the interstitium dictates that large particles (roughly larger than 0.1 μm) will have more difficulty to pass through the interstitium and will remain at the site of injection to a large, almost complete extent. Smaller particles can mi-

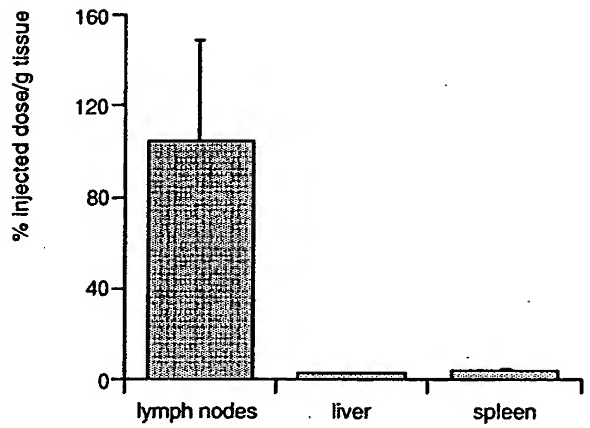

Fig. 3. Comparison of uptake of radiolabelled liposomes in percentage of injected dose per gram tissue in regional lymph nodes, liver and spleen. A single dose of liposomes (EPC:EPG:Chol, 10:1:4 molar ratio, mean diameter 0.07 μm, 2.5 μmol lipid) was injected s.c. into the dorsal side of the foot of rats. Values represent the mean percentage±S.D. of four animals.

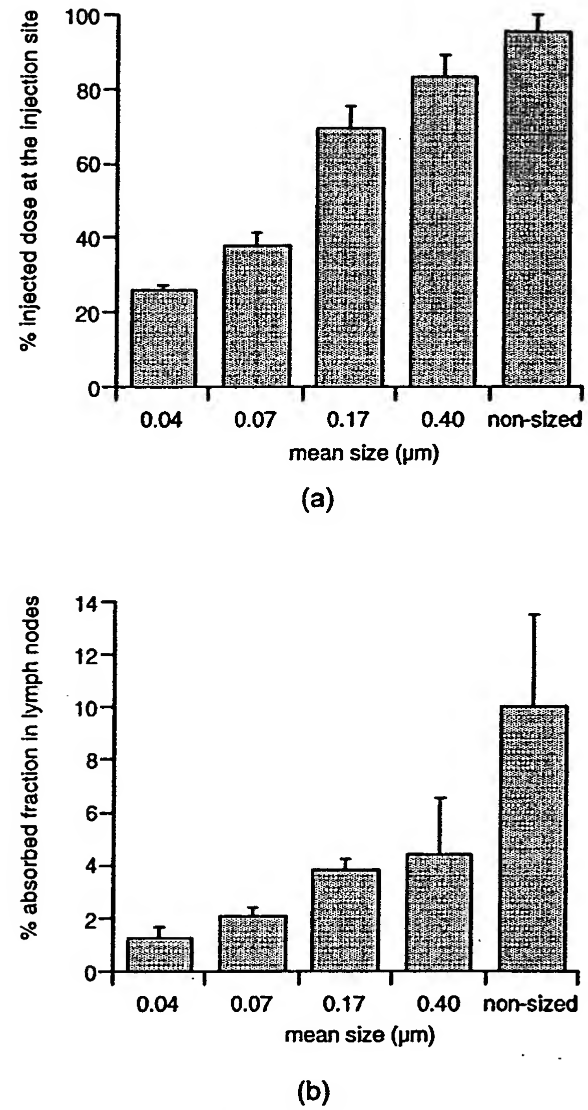

Fig. 4. Influence of size on lymphatic absorption and lymph node uptake of s.c. administered liposomes. A single dose of liposomes (EPC:EPG:Chol, 10:1:4 molar ratio, 2.5 μmol lipid) of varying size was injected s.c. into the dorsal side of the foot of rats. Levels of radioactivity were determined 52 h post-injection. (A) Percentage of injected dose recovered from the s.c. injection site. (B) Percentage of the lymphatically absorbed fraction recovered from regional lymph nodes (relative lymph node uptake). Values represent the mean percentage±S.D. of four animals.

grate through aqueous channels in the interstitium and therefore have better access to the lymphatic systems [18]. However, it should be taken into account that even after injection of liposome dispersions with a small mean size a substantial fraction of the injected dose remains at the injection site. Typically, after s.c. injection of small liposomes (mean size about 70 nm) on the dorsal side of the foot about 40% of the injected dose remains at the injection site (Fig. 4A). A possible explanation for the incomplete absorption might be the heterogeneous size distribution of the liposome dispersion with liposomes substantially larger than the mean size of 70 nm being retained at the site of injection. Another explanation relates to an alteration of the interstitial pressure during the initial period of absorption. As lymphatic absorption from the injection site may be the result of an elevated interstitial pressure caused by the injection itself, lymphatic absorption may stop when the interstitial pressure is normalized. A third possibility is aggregation of liposomes at the site of injection resulting in the formation of large aggregates that will remain at the site of injection.

Lymph node localization is much less dependent on liposome size. Lymph node uptake of small liposomes (mean size 0.04 μm) was found to be almost equal to the uptake of large, non sized liposomes. In fact, when expressed as the percentage of the lymphatically absorbed fraction (relative lymph node uptake), lymph node uptake was found

to be much higher for large liposomes than for small liposomes (Fig. 4B) [14]. Apparently, large liposomes are retained more efficiently by lymph nodes than small liposomes, most probably because large liposomes are filtered out more efficiently than smaller liposomes. Also, in view of the outcome of studies on the interaction between liposomes and macrophages, larger particles may be phagocytosed more efficiently by lymph node macrophages than smaller liposomes [19].

2.2. Influence of liposome composition

Lipid composition of liposomes is another factor potentially influencing lymphatic disposition of s.c. administered liposomes. In a systematic study on the influence of liposome composition on lymphatic disposition of s.c. injected liposomes it was demonstrated that both lymphatic absorption and lymph node uptake are independent of the presence of a negatively charged lipid, the presence of cholesterol, and bilayer fluidity as compared to liposomes composed of EPC:EPG:Chol (Fig. 5A) [[14]]. Also lymph node localization was found to be independent of these lipid composition related parameters. Only incorporation of phosphatidylserine (PS) in the bilayers of EPC:EPG:Chol liposomes resulted in significantly increased lymph node uptake (Fig. 5B). It has been shown that PS-exposure serves as a signal for triggering recognition by macrophages [20]. Presumably, the substantially increased lymph node uptake of PS-containing liposomes can be attributed to the same mechanism.

2.3. Influence of lipid dose

Results of a study on the influence of lipid dose demonstrated that both the percentage of injected dose of liposomes remaining at the injection site as well as the percentage of dose taken up in regional lymph nodes is independent of the injected dose at a dose range of 10^1–10^4 nmol lipid per rat. The absolute amount of lipid recovered from the injection site appeared to be linearly correlated to the injected dose (Fig. 6A). The same observation applies for lymph node localization. The absolute amount of liposomal label recovered from lymph nodes in-

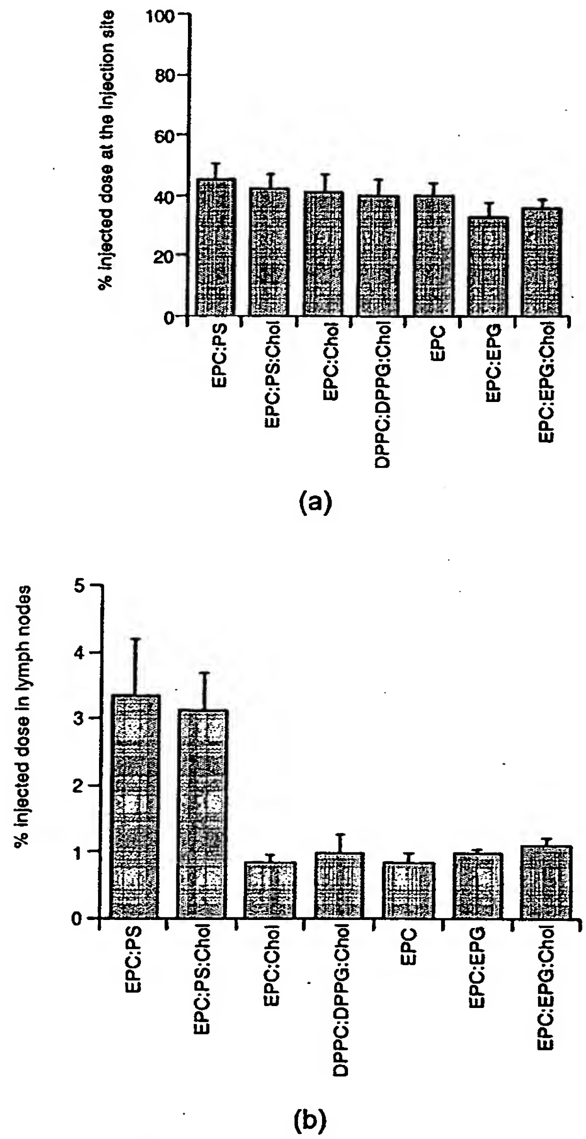

Fig. 5. Influence of liposome composition on lymphatic absorption and lymph node uptake of s.c. administered liposomes. A single dose of small liposomes (mean diameter 0.07 μm, 2.5 μmol lipid) of varying composition was injected s.c. into the dorsal side of the foot of rats. Levels of radioactivity were determined 52 h post-injection. (A) Percentage of injected dose recovered from the s.c. injection site. (B) Percentage of injected dose recovered from regional lymph nodes. Values represent the mean percentage±S.D. of four animals.

creased almost linearly with increasing dose (Fig. 6B). Moreover, repeated injections (one injection given daily over four subsequent days) of similar

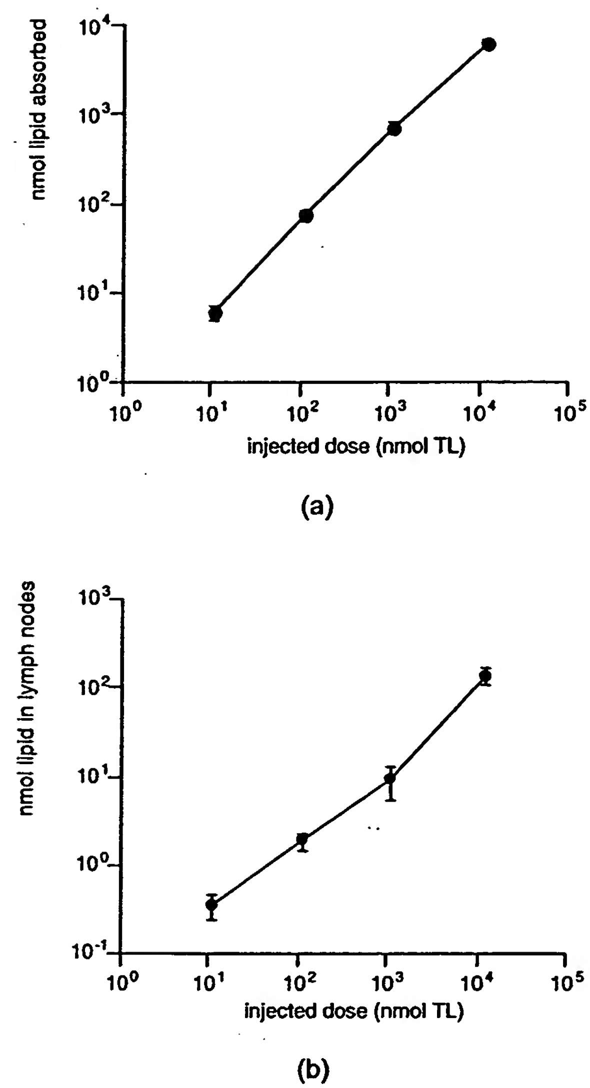

Fig. 6. Influence of lipid dose on lymphatic absorption and lymph node uptake of small liposomes. A single dose of small (0.10 μm) liposomes (EPC:EPG:Chol, 10:1:4 molar ratio) was injected s.c. into the dorsal side of the foot of rats in escalating lipid dose (i.e. 10, 10^2, 10^3, 10^4 nmol lipid). Levels of radioactivity at the site of injection and in regional lymph nodes were determined 52 h post-injection. (A) Absolute amount of lipid taken up from the s.c. injection site. (B) Absolute amount of lipid recovered from regional lymph nodes. Values represent the mean percentage±S.D. of four animals.

liposomes also resulted in a linear dose-dependency of the accumulation of liposomes in regional lymph nodes [14]. Apparently, lymphatic absorption and lymph node uptake are not saturated at the dose range tested.

2.4. Influence of surface modification

2.4.1. Surface modification with polyethylene(glycol) (PEG)

The incomplete lymphatic absorption of s.c. administered particles as described above may be the result of interactions between the particle surface and components of the interstitium inducing formation of larger particles that are not taken up by the lymphatic capillaries but will remain at the site of injection. It has been suggested that it might be possible to increase lymphatic drainage by applying the concept of steric stabilization [21]. Coating the liposomal surface with a sterically stabilizing, hydrophilic layer, may reduce nonspecific interactions of particles with the interstitial surroundings and inhibit the formation of particle structures too large for lymphatic absorption. Moreover, the increased hydrophilicity of particles with a sterically stabilized surface may allow improved migration through the aqueous channels of the interstitium possibly resulting in increased lymphatic absorption. Upon reaching a lymph node, sequestration of liposomes may occur by either phagocytosis or by simple mechanical filtration. As steric stabilization of the liposomal surface with PEG reduces adsorption of proteins to the liposomal surface, such liposomes would tend to avoid uptake by macrophages ('stealth' effect). Therefore, PEG-coating of the liposomal surface may have a negative effect on lymph node uptake. However, as hypothesized above, lymphatic absorption from the s.c. injection site may have increased as a result of the steric stabilization effect and, therefore, the net amount of liposomes taken up by the lymph nodes may be higher despite the stealth effect.

The influence of a sterically stabilized surface on the lymphatic absorption of liposomes after s.c. injection was first studied by Allen et al. [15]. Following s.c. administration, only small (< 0.1 μm) non-coated and PEG-coated liposomes were detected in the circulation. PEG-coated liposomes yielded higher blood levels when compared to non-coated liposomes. Additionally, PEG-coated liposomes resulted in lower lymph node uptake, as compared to

non-coated liposomes. In a more detailed study on the effect of the PEG-coating, the actual amount of liposomes remaining at the injection site and lymph node uptake of PEG-coated and non-coated liposomes was determined [22]. It was found that PEG-coated liposomes are slightly better absorbed than non-coated liposomes (Fig. 7A). Additionally, lymph node uptake of PEG-coated liposomes was hardly affected compared to non-coated liposomes (Fig. 7B).

The question arises why the PEG-sterically stabilized surface does not exert a strong effect on lymphatic absorption and lymph node uptake as is hypothesized above. Apparently, factors other than the presence of a steric barrier are more important in determining lymphatic absorption from the s.c. in-

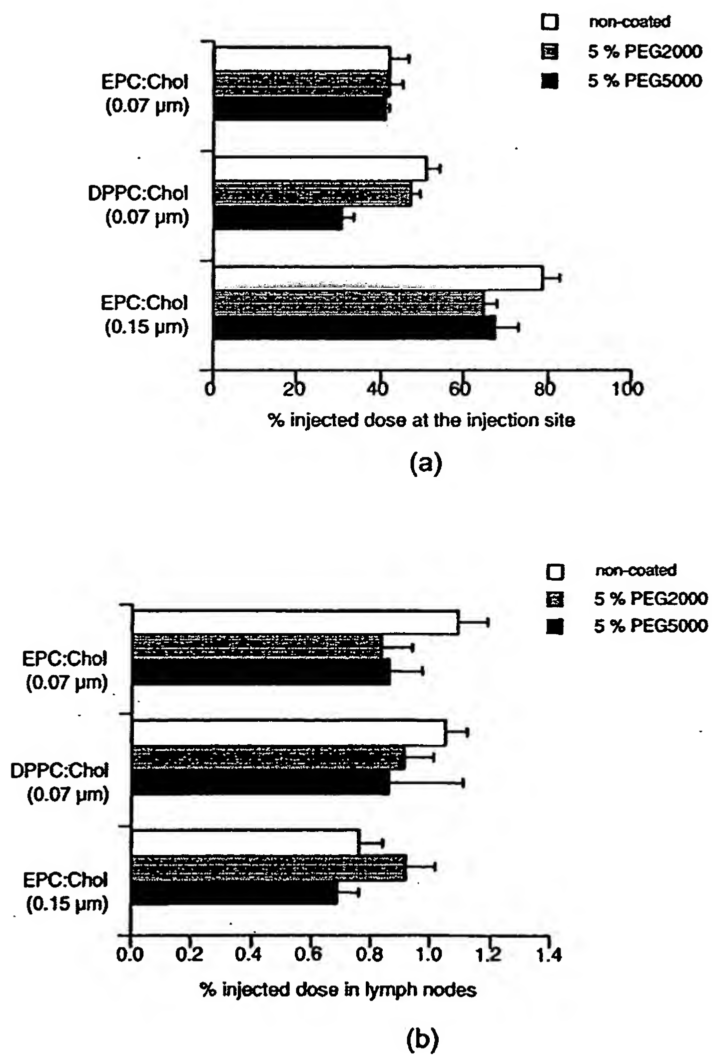

Fig. 7. Effect of PEG-coating on the lymphatic absorption and lymph node uptake of s.c. administered liposomes. A single injection of PEG-coated liposomes and non-coated liposomes (EPC: Chol (2:1 molar ratio) or DPPC: Chol (2:1 molar ratio)), was given s.c. into the dorsal side of the foot of rats. Levels of radioactivity were determined 52 h post-injection. (A) Percentage of injected dose recovered from the s.c. injection site. (B) Percentage of injected dose recovered from regional lymph nodes. Values represent the mean percentage±S.D. of four animals.

jection site. Additionally, the observation that lymph node uptake is only slightly affected by PEG-mediated steric stabilization strongly suggests that macrophage uptake is not the only important mechanism of lymph node localization of s.c. administered liposomes.

2.4.2. Surface modification with ligands

Considering the relatively high degree of lymphatic absorption of small liposomes (< 0.1 μm) and thus the large amounts of liposomes passing through the lymph nodes, the absolute uptake of liposomes by regional lymph nodes is low (about 1–2% of the injected dose). Apparently, most of the liposomes that are absorbed from the s.c. injection site (up to 60% of the injected dose) pass through the lymphatics without being captured in lymph nodes. Attempts to enhance lymph node uptake by attaching ligands to the liposomal surface have been reported occasionally. Liposomes coated with non-specific human antibodies injected s.c. showed a modestly increased lymphatic absorption and lymph node uptake compared to liposomes without the coating [23]. The influence of attachment of saccharide derivatives to the liposomal bilayer on lymphatic disposition has been investigated as well. Saccharide modified liposomes showed enhanced absorption from the injection site and enhanced lymph node uptake compared to control liposomes [24].

More recently a study has been performed with liposomes bearing Fab′ fragments (immunoliposomes) directed against the HLA-DR surface marker which is expressed on monocytes/macrophages and activated CD4+ T-lymphocytes. Anti-HLA-DR liposomes were s.c. injected into the upper back of mice and lymph node uptake was studied in regional lymph nodes, liver and spleen. When compared to conventional liposomes, the s.c. administration of anti-HLA-DR immunoliposomes resulted in a up to 3-fold higher accumulation in regional lymph nodes [25].

2.4.3. Surface modification with biotin

A novel method to enhance lymph node uptake of s.c. injected liposomes has recently been published by Phillips et al. [26]. They combined the ability of the lymphatic system to absorb small s.c. injected liposomes with the preferential uptake of larger liposomes in regional lymph nodes. The newly developed system is comprised of a s.c. injection of biotin-coated liposomes, followed by an adjacent s.c. injection of avidin. As the avidin moves through the lymphatic vessels, it causes aggregation of biotin-coated liposomes that are also in the progress of migrating through lymphatic vessels. The aggregated liposomes become entrapped in the next encountered lymph node. Lymph node uptake after injection of biotin-liposomes and avidin proved to be much higher (up to 14% of the injected dose), than after injection of biotin-liposomes without the avidin (about 2% of the injected dose).

2.5. Influence of the anatomical site of injection

We recently reported that uptake in regional lymph nodes is low after s.c. injection into the flank whereas injection of similar liposomes into the dorsal side of the foot or into the footpad results in much higher lymph node uptake (Fig. 8A and B) [27]. After s.c. injection into the flank, liposomes remain almost completely at the site of injection, whereas after injection into the foot, 40–50% of the injected dose is taken up from the injection site The observed site-dependent disposition of liposomes may be explained as follows. The absorption of small colloid particles from interstitial tissue into the lymphatics is a passive process, occurring naturally as lymph is formed. The rate of lymphatic absorption is therefore related to the lymph flow. When the pressure in interstitial tissue exceeds that within the lymphatic capillaries, intercellular junctions in the lymphatic capillaries open up and lymph flow and thus lymphatic absorption of interstitial fluid increases [28–30]. As injected fluid may cause an increase in interstitial pressure, the injection itself may be an important determinant of the efficiency of the lymphatic drainage process. The observed site-dependent disposition is attributed to differences in the structural organization of the s.c. tissue at different anatomical sites. In rats, hardly any s.c. fat-layer is present at the footpad and at the dorsal side of the foot, whereas at the flank a s.c. layer of loose adipose tissue is found. As a result of the limited interstitial space at both s.c. sides of the foot, the injected dispersion may induce a rise in local interstitial pressure. In contrast, s.c. injection of liposomes

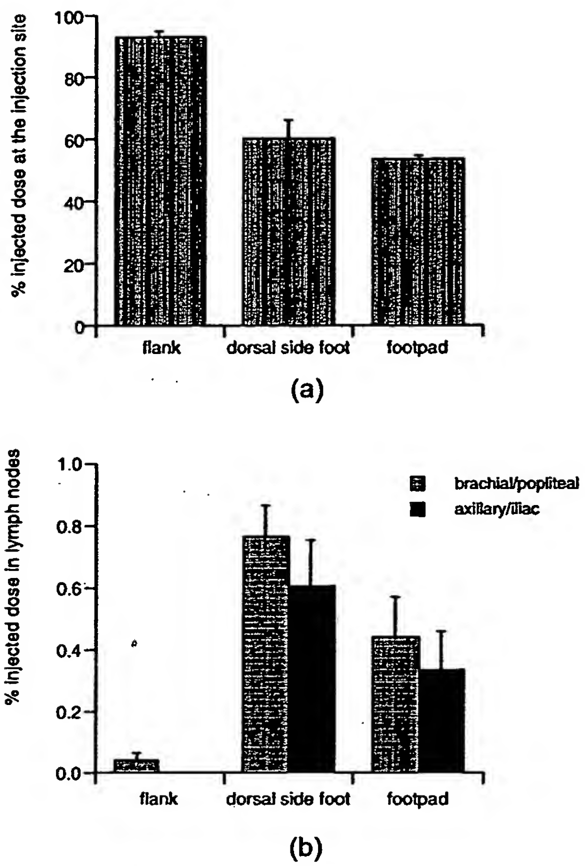

Fig. 8. Influence of anatomical site of injection on lymphatic absorption and lymph node uptake of small liposomes. A single dose of radiolabelled liposomes (EPC:EPG:Chol, mean diameter 0.10 μm, 2 μmol lipid) was injected s.c. into the flank, into the dorsal side of the foot and into the footpad of rats. Levels of radioactivity at the site of injection and in regional lymph nodes were determined 52 h post-injection. (A) Percentage of injected dose recovered from the s.c. injection site. (B) Percentage of injected dose recovered from regional lymph nodes. Values represent the mean percentage of injected dose remaining at the site of injection±S.D. of four animals.

into the flank will not cause such an increase in interstitial pressure as the injected fluid will be able to spread over a large area of s.c. adipose tissue. Therefore, at the flank lymphatic absorption of interstitial fluid containing liposomes will not be driven by the injection and consequently will be much less.

The observed site-dependent kinetics of lymphatic absorption of s.c. administered liposomes appears to be species related. Results of a study in guinea pigs revealed that lymphatic absorption and lymph node uptake after s.c. injection into the flank of guinea pigs was much higher compared with liposomes injected in the flank of rats [27]. As the skin of guinea pigs is known to be very tight compared to rat, it might be possible that the injection results in increased interstitial pressure in the skin of guinea pigs which favors lymphatic absorption from the site of injection. These results indicate that the anatomical site of injection should be carefully considered when developing approaches involving lymphatic drug delivery by means of s.c. administered liposomes.

In a few reports the effect of massage of the s.c. injection site has been reported to stimulate lymphatic absorption of s.c. injected liposomes. Massage of the local injection site induces elevated pressure at the injection site and thus enhanced lymphatic absorption of interstitial fluid including injected material. Accordingly, lymphatic absorption of liposomes increases substantially when the s.c. injection site is manually massaged [31,32].

3. Lymph node localization

Phagocytosis by macrophages is generally accepted as the major mechanism of uptake of liposomes in lymph nodes. Liposomes taken up by lymph node macrophages after s.c. injection have been visualized by light and electron microscopy (Fig. 9) [33,34]. In line with the microscopic observations on macrophage uptake, inclusion of phosphatidylserine (PS) in the liposomal bilayers, a strong signal stimulating macrophage uptake [[20]], substantially enhances lymph node uptake. However, on the other hand, coating of small (0.1 μm) liposomes with PEG, which has proven to oppose macrophage uptake, hardly affects lymph node uptake, suggesting that phagocytosis by macrophages is not the only mechanism involved in lymph node uptake of liposomes [15,22,35,36].

The role of macrophages in lymph node uptake of liposomes was confirmed in a study in rats in which lymph nodes were selectively depleted of macrophages. To achieve depletion of macrophages, clodronate-containing liposomes were s.c. injected in the footpad [37,38]. Six days after injection of clod-

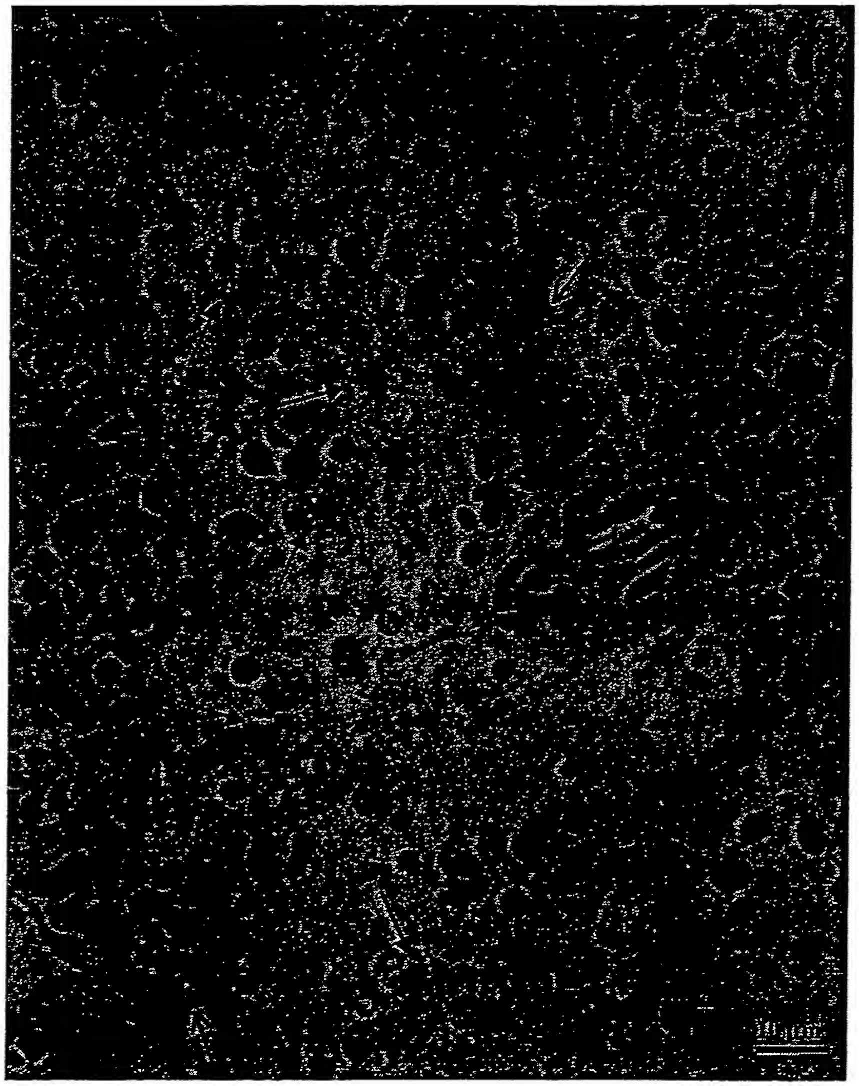

Fig. 9. Lightmicrograph of 1 μm resin silver-enhanced section of a popliteal lymph node isolated 6 h after s.c. injection of small (0.10 μm) gold-liposomes composed of EPC:EPG:Chol (molar ratio 10:1:4) into the foot. Paracortical sinus; macrophages (closed arrows) and endothelial cells (open arrows) containing colloidal gold particles.

ronate-containing liposomes, small (mean diameter 0.1 μm) and large, non-sized, radiolabelled liposomes were s.c. injected at the dorsal side of the same foot. Pretreatment with liposomal clodronate resulted in a drastic reduction of lymph node uptake of large as well as small liposomes. The reduced lymph node uptake confirmed that phagocytosis by macrophages plays an important role in nodal uptake of liposomes [33].

The effect of macrophage depletion on lymph node uptake was also described for PEG-coated liposomes. As surface modification of liposomes with PEG is known to oppose phagocytosis by macrophages one would expect that the depletion of macrophages has only a minor effect on lymph node uptake of PEG-coated liposomes. However, in contrast to this hypothesis macrophage depletion has a strong negative effect on the degree of uptake of

PEG-coated liposomes. Moreover, under non-depleted conditions, large numbers of colloidal gold particles were observed in macrophages after administration of PEG-coated gold-liposomes [33]. These results, pointing to efficient uptake of PEG-coated liposomes by macrophages, can not be attributed to a failure to achieve effective steric stabilization, as the PEG-coated liposomes proved to be long-circulating after reaching the blood circulation. It was hypothesized that the initial mechanism of lymph node uptake may be the result of mechanical depth filtration in the meshwork of reticular cells in the lymph node. Because of the slow progression of the liposomes through the lymph node, enough time might be available for an effective interaction of the PEG-liposomes with phagocytes and, given enough time, even PEG-coated liposomes will be taken up by macrophages.

4. Diagnostic and therapeutic applications

4.1. Imaging

The presence of lymph node metastases is often a prognostic factor in neoplastic diseases. As early as 1979, ^{99m}Technetium-liposomes were suggested as carriers of contrast agents for lymph node visualization [39]. However, since then only a few reports on the use of s.c. injected liposomes as lymphotropic imaging agents appeared [40–42]. Possibly, lymph node uptake of radio-labeled liposomes is not sufficient for adequate imaging. Moreover, the growth of metastatic tumor cells in regional lymph nodes may cause blockage of normal lymph flow and destruction of the internal structure of regional lymph nodes, thus affecting lymphatic absorption and lymph node uptake of s.c. administered liposomes [43,44].

At present non-enhanced local CT and MR imaging are widely practiced imaging techniques for lymph node examinations. Unfortunately, non-enhanced imaging techniques are useful only when relatively large lymph nodes are involved [45]. Attempts have been made to develop liposomes that enhance MR-signal. Liposome-encapsulated gadolinium was found to be a suitable lymphography contrast agent in animal experiments as relatively high concentration were detected in regional lymph nodes after s.c. injection [31,46]. In another MR-imaging study it was found that both PEG-coated and dextran-coated liposomes with surface-incorporated gadolinium enhance MR-signal compared to non-coated liposomes [47]. In a subsequent study, it was demonstrated that visualization of lymph nodes with PEG-coated liposomes is achieved within minutes after s.c. administration. Moreover, coating of the liposomal surface with PEG increases the nodal enhancement 3–3.5-fold compared to the non-coated liposomes [48].

4.2. Delivery of anti-tumor drugs

The lymphatic system is an important pathway for the spread of metastases [2]. In spite of radical surgery, a large number of cancer patients dies because of the formation of metastases. In clinical practice systemic adjuvant chemotherapy is usually given after surgical debulking of the tumor burden to prevent and eliminate lymphatic metastases. Nevertheless, tumor recurrence is frequent as tumor cells residing in the lymphatic system are poorly accessible for i.v. administered anti-tumor agents. The observation on relatively high localization of interstitially injected liposomes in regional lymph nodes provides a rationale for therapy of lymph node metastases by s.c. administration of anti-tumor drugs encapsulated in liposomes [11,49–54]. Indeed, liposome-encapsulated anti-tumor drugs have been shown to suppress lymphatic metastasic growth more effectively than the free, non-encapsulated drug [55–58]. The relatively high therapeutic efficacy of s.c. administered liposomes can be explained to two different mechanisms: (i) liposomes are efficiently captured in regional lymph nodes after being absorbed from the injection site into lymphatic vessels. Sustained release of drug from intranodal liposomes may provide relatively high and prolonged concentrations in lymph node tissue; (ii) sustained release of drug from liposomes remaining at the injection site provides low drug concentrations at the interstitial site for prolonged periods of time. Part of the released drug may be absorbed by lymphatic vessels and transported to regional lymph nodes, thus providing low but lasting concentrations in lymph node tissue.

Subcutaneous treatment with liposome-encapsulated anti-tumor drugs is hampered by several limitations. One limiting factor is that lymphatic absorption of liposomes after s.c. injection is not complete. Although liposomes have the potential to protect surrounding tissue against local toxicity of the encapsulated anti-tumor drug, leakage of drug may cause serious tissue damage, with the anti-tumor drug doxorubicin as an well-known example [59–62]. Another limiting factor is that the growth of metastatic tumor cells in regional lymph nodes may cause blockage of normal lymph flow and destruction of the internal structure of regional lymph nodes, thus affecting lymphatic absorption and lymph node localization of s.c. administered liposomes [43,44]. Moreover, lymph nodes with malignant cells may contain less macrophages, thus reduced liposome uptake should be anticipated. Therapeutic efficacy may also be limited by insufficient drug release from the liposomes in the lymph node. Another concern involves the observation that liposomal anti-tumor drugs, such as liposomal doxorubicin, can deplete macrophages [63]. As macrophages are capable of controlling metastatic growth in vivo [64], such macrophage depleting effect may contribute to treatment failure.

The potential of local administration of anti-tumor drugs encapsulated in liposomes has recently been studied in a clinical setting. In a pilot study, eight breast cancer patients received an injection of mitoxantrone free or encapsulated in liposomes in the proximity of the tumor. Two days after administration the tumor and the injection site were dissected by operation. The results demonstrated much higher concentrations of mitoxantrone in regional lymph nodes in case of administration of the drug encapsulated in liposomes [65]. This approach may prove to be useful in the prevention and early treatment of lymph node metastasis in breast cancer.

5. Conclusion

This review is focussed on the utility of liposomes for lymphatic targeting after s.c. administration. A s.c. administered carrier system for the delivery of diagnostic and therapeutic agents to regional lymph nodes should combine two major characteristics: efficient absorption into the lymphatic system and high lymph node uptake. When expressed as percentage of injected dose per gram tissue, lymph node uptake of small liposomes in regional lymph nodes is relatively high compared to uptake in spleen and liver, the natural target organs for liposomes circulating in the bloodstream. Considering the fact that liposomes will encounter lymph nodes only once when passing through on their way to the blood circulation and do not have, as in the case of liver and spleen, the possibility of multiple passage, lymph node uptake of liposomes is apparently an efficient process. However, the absolute amount of lymph node uptake is low as only a few percent of the injected dose localizes in regional lymph nodes. To enhance lymph node uptake attempts are made to target nodal tissue. Altogether, the results of studies on liposomes for lymphatic targeting after subcutaneous administration suggest that s.c. administration of liposome-encapsulated drugs may provide a valuable means of concentrating diagnostic, therapeutics and immunomodulatory agents in regional lymph nodes.

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Liposomes to target the lymphatics by subcutaneous administration

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Abstract

Liposomes have been proposed as carriers for the delivery of therapeutic and diagnostic agents to the lymphatic system. Subcutaneous (s.c.) injection is the route of administration most extensively studied for this purpose. Decisive factors influencing lymphatic absorption and lymph node uptake of s.c. administered liposomes are liposome size and the anatomical site of injection. Generally, other factors such as lipid composition, charge and the presence of a hydrophilic PEG-coating on the liposome surface do not substantially affect lymphatic absorption and lymph node uptake of s.c. administered liposomes. Studies on the intranodal fate of liposomes demonstrate that phagocytosis by macrophages is the most important mechanism for lymph node uptake of liposomes. The observation of relatively high uptake of liposomes in regional lymph nodes after s.c. administration has stimulated research on lymphatic targeting of liposomes for diagnostic and therapeutic applications.

Keywords: Subcutaneous; Targeting; Liposomes; Lymphatics

Contents

Abbreviations: Chol, cholesterol; DPPC, distearoylphosphatidylcholine; DPPG, dipalmitoylphosphatidylglycerol; EPC, egg-phosphatidylcholine; EPG, egg-phosphatidylglycerol; i.m., intramuscular; i.p., intraperitoneal; i.v., intravenous; PEG, poly(ethyleneglycol); PS, phosphatidylserine; s.c., subcutaneous

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